REMARKS

In the claims

Claims 1–2, and 5–12 are currently under examination with claims 3, 4, and 13–18 withdrawn due to restriction/election.

Claim amendments

The claims are amended to recite language in accordance with conventional US practice. It is courteously submitted that the claim amendments do not raise new matter.

Specification

The Office Action alleges that the format of the ABSTRACT does not comply with the guidelines stated under M.P.E.P. §608.01(b). Applicants respectfully disagree. The Patent Office is requested to review page 26 of the specification, wherein an ABSTRACT is provided in a separate page. It is submitted that the enclosed ABSTRACT fully conforms with the guidelines stated under subsections (a)-(c) under §608.01(b). Clarification of the objection is respectfully requested.

Regarding the use of subtitles, Applicants prefer not to use the PTO's suggested subtitles since these are not mandatory. Withdrawal of the objection is respectfully requested.

Rejections under 35 U.S.C. §112, second paragraph

The rejection of claim 1 is moot in view of its amendment. Withdrawal of the rejection is respectfully requested.

Claim 11 stands objected regarding the use of FLAG/anti-FLAG antibody. It is courteously submitted that the use of registered mark [®] in the claim constitutes a typographical error, and the specification has been duly amended to be in conformance

with the claims. It is submitted that the meaning of FLAG (or a FLAG peptide), as recited herein, is well-defined in the area of biology. The Patent Office is courteously requested to review the enclosed scientific ABSTRACTS, all of which corroborate with the entirety of disclosure contained in Applicants' instant specification relating to the expressed recitation and use of such compounds.

The Patent Office is respectfully requested to withdraw the pending rejections under 35 U.S.C. §112, second paragraph.

Rejection under 35 U.S.C. §102(b)

Claims 1–2, and 5–11 stand rejected under 35 U.S.C. §102(b) as being anticipated by Nicolson et al (US 4,859,581). Applicants respectfully traverse this rejection.

The Patent Office's contention that Nicholson uses "donor and acceptor" compound in a manner that is commensurate with Applicants' claimed invention is grossly misplaced. For example, the disclosure in col. 11, lines 4–9 of Nicholson merely discloses the use of 'fluorescein-labeled HS for a rapid analysis of degradation fragments on HPLC equipped with a flow fluorescence detector." These are not donor-acceptor compounds which are in close proximity to one another. As one of ordinary skill in the art can readily attest to, this rather static method of detection does not recite the specific method claimed herein. Nowhere does the cited reference teach or suggest a method for detecting endoglycosidase activity by utilizing a substrate which is directly or indirectly labeled with a first donor compound and with a second acceptor compound, and in that the amount of intact substrate is determined by measuring a signal emitted by the acceptor compound, this signal resulting from a transfer, via a close proximity effect, between the donor and the acceptor compounds. See, Applicants' claim 1.

Nicholson further describes a method for determining endoglycosidase activity for the detection of "carcinogenic malignancies" comprising hydrolyzing a substrate linked to a solid phase support. See, col. 1, lines 9–15 and col. 4, lines 49–55. The cited reference discloses that the hydrolysis of the substrate yields soluble labeled products, which are then separated prior to detection. See, col. 8, lines 30–36; col. 9, lines 3–10. Methods for practicing the separation step are also provided. See, col. 9, lines 21–27. At col. 10, lines

23–28, Nicholson expressly teaches that "the separation of the reaction products from the substrates based on their size is required for the heparanase assay." This limitation is also expressly claimed by Nicholson, for example, in claim 1 and claims 7–10. In contrast, Applicants' claimed invention does not necessitate size-resolution. Thus, it is clearly different.

Since not all elements of Applicants' claims are recited in the cited reference, it is courteously submitted that Nicholson can neither anticipate nor render obvious the instantly claimed subject matter. Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §103(a)

The contention that the aforementioned reference of Nicholson in view of Bazin (Spectrochimica Acta, **vol. 57**, pages: 2197–2211, **2001**) renders obvious the instantly claimed subject matter is respectfully traversed.

Bazin teaches the use of Europium crytate and allphycocyanin as donor and acceptor compounds in homogenous time-resolved fluorescence (HTRF) assays. See, §1.2 of the cited reference. As outlined in §3.1 at page 2205, it is taught that the substrates used in these assays are proteins, and the assay is primarily used in the measurement of protein-protein interactions. §3.2 and §3.3 provide further applications of the disclosed HTRF assay. In this context, §3.2.2 of the cited reference describes the use of this method in a *protease assay* wherein "a peptide containing a sequence recognized by a protease is labeled on one side with XL665 (allphycocyanin) and on the other side with biotin [and] in presence of the protease, which cleaves the peptide, the signal is measured." Nothing in the cited reference would lead a skilled artisan to employ Bazin's peptidase assay for the assessment of the activity of a glycosidase enzyme, as taught by Nicholson. As one of ordinary skill in the art can readily attest to, the structural components (for *e.g.*, substrates and their cleavage sites) and the functionality (for *e.g.*, enzymatic activity, agonists, inhibitors etc.) of Bazin's peptidases are distinct from the enzymes claimed by Nicholson. See, the enclosed references.

Applicants respectfully submit that Bazin's methods, which are directed to the assessment of enzymatic activity using peptide substrates are distinct from the claimed

invention, which is based on non-protein substrates such as polysaccharides. As one of ordinary skill in the art can readily attest to, the structural features of peptide substrates are distinct from their sugar counterparts (for e.g., heparin sulfate of the instant invention). For example, peptide substrates have predictable cleavage sites and predictably substituted derivatives with respect to the spatial placement of donor and acceptor molecules.

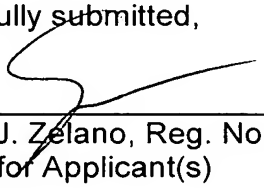
It is courteously submitted that Bazin fails to provide any hint or suggestion to one of ordinary skill that the peptide-peptidase (substrate-enzyme) system could be applied in a complex and the challenging assay technique such as one described by the instant invention. Absent such guidance, one of ordinary skill in the art would not be motivated to reformulate the cited references to arrive at the instant invention. Therefore, a combination of Nicholson and Bazin fails to render obvious the subject matter of the instant invention.

Withdrawal of the rejection is respectfully requested.

In view of the above remarks, favorable reconsideration is courteously requested. If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

No fees are believed to be due with this response; however, the Commissioner is hereby authorized to charge any fees associated with this response to Deposit Account No. 13-3402.

Respectfully submitted,



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Date: January 16, 2007
Encl:

Reference publications 1 and 2
Abstracts

Protease

From Wikipedia, the free encyclopedia
(Redirected from Peptidase)

Proteases (proteinases, peptidases, or proteolytic enzymes) are enzymes that break peptide bonds between amino acids of proteins. The process is called *peptide cleavage*, a common mechanism of activation or inactivation of enzymes, especially those involved in blood coagulation or digestion. They use a molecule of water for this and are thus classified as hydrolases.

Contents

- 1 Classification
- 2 Occurrence
- 3 Inhibitors
- 4 Degradation
- 5 Protease research
- 6 References
- 7 External links

Classification

There are currently six classes of proteases:

- Serine proteases
- Threonine proteases
- Cysteine proteases
- Aspartic acid proteases (*e. g.*, plasmepsin)
- Metalloproteases
- Glutamic acid proteases

the threonine and glutamic acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the character of a polarized peptide bond (serine, cysteine and threonine peptidases) or a water molecule (aspartic acid, metallo- and glutamic acid peptidases) nucleophilic so that it can attack the peptide carbonyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine or threonine as a nucleophile.

Occurrence

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase activating cascade). Peptidases can break either specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components; it can be an activation of a function or it can be a signal in a signalling pathway.

Proteases are also a type of exotoxin, which is a virulence factor in bacteria pathogenesis. Bacteria exotoxic proteases destroy extracellular structures.

Inhibitors

The function of peptidases is inhibited by protease inhibitor enzymes. Examples of protease inhibitors are the class of serpins (*ser*ine *p*rotease or *p*eptidase *in*hibitors), incorporating alpha 1-antitrypsin. Other serpins are complement 1-inhibitor, antithrombin, alpha 1-antichymotrypsin, plasminogen activator inhibitor 1 (coagulation, fibrinolysis) and the recently discovered neuroserpin.

Natural protease inhibitors include the family of lipocalin proteins, which play a role in cell regulation and differentiation. Lipophilic ligands, attached to lipocalin proteins, have been found to posess tumor protease inhibiting properties. The natural protease inhibitors are not to be confused with the protease inhibitors used in antiretroviral therapy. Some viruses, with HIV among them, depend on proteases in their reproductive cycle. Thus, protease inhibitors are developed as antiviral means.

Degradation

Proteases, being themselves proteins, are known to be cleaved by other protease molecules, sometimes of the same variety. This may be an important method of regulation of peptidase activity.

Protease research

The field of protease research is enormous. Barrett and Rawlings estimated that approximately 8000 papers related to this field are published each year.

References

- Barrett A.J., Rawlings ND, Woessner JF. *The Handbook of Proteolytic Enzymes*, 2nd ed. Academic Press, 2003. ISBN 0-12-079610-4.
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- Southan C. *A genomic perspective on human proteases as drug targets.* Drug Discov Today 2001;6:681-688.
- Hooper NM. *Proteases in Biology and Medicine.* London: Portland Press, 2002. ISBN 1-85578-147-6.
- Puente XS, Sanchez LM, Overall CM, Lopez-Otin C. *Human and Mouse Proteases: a Comparative Genomic Approach.* Nat Rev Genet 2003;4:544-558.
- Ross J, Jiang H, Kanost MR, Wang Y. *Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships.* Gene 2003;304:117-31.
- Puente XS, Lopez-Otin C. *A Genomic Analysis of Rat Proteases and Protease Inhibitors.* Genome Biol 2004;14:609-622.

External links

- Merops - the peptidase database
- List of protease inhibitors

Enzymes [hide]

Active site - Binding site - Catalytically perfect enzyme - Coenzyme - Cofactor - EC number - Enzyme catalysis - Enzyme kinetics - Enzyme inhibitor - Michaelis-Menten kinetics

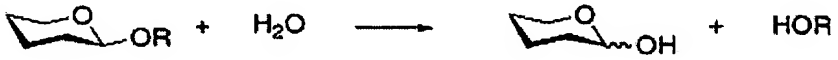
Oxidoreductases/list - **Transferases**/list (DNA methyltransferase, phosphotransferase, polymerase, DNA, I, II, III, RNA, I, II, III, Primase) - **Hydrolases**/list (nuclease, esterase, phosphodiesterase, lipase, **protease**, DNA glycosylases) - **Lyases**/list (carboxy-lyases, dehydratase) - **Isomerases**/list (topoisomerase, gyrase, topoisomerase IV) - **Ligases**/list (aminoacyl tRNA synthetase)

Categories: EC 3.4 | Posttranslational modification

Glycoside hydrolase

From Wikipedia, the free encyclopedia

Glycoside hydrolases (also called **glycosidases**) catalyze the hydrolysis of the glycosidic linkage to generate two smaller sugars. They are extremely common enzymes with roles in nature including degradation of biomass such as cellulose and hemicellulose, in anti-bacterial defense strategies (eg lysozyme, in pathogenesis mechanisms (eg viral neuraminidases) and in normal cellular function (eg trimming mannosidases involved in N-linked glycoprotein biosynthesis). Together with glycosyltransferases, glycosidases form the major catalytic machinery for the synthesis and breakage of glycosidic bonds.



Contents

- 1 Classification
  - 1.1 Sequence-based classification
- 2 Mechanism
  - 2.1 Inverting glycoside hydrolases
  - 2.2 Retaining glycoside hydrolases
- 3 Nomenclature and examples
- 4 Uses
- 5 See also
- 6 References

Classification

Glycoside hydrolases are classified into EC 3.2.1 as enzymes catalyzing the hydrolysis of O- or S-glycosides. Glycoside hydrolases can also be classified according to the stereochemical outcome of the hydrolysis reaction: thus they can be classified as either *retaining* or *inverting* enzymes.[1] Glycoside hydrolases can also be classified as exo or endo acting, dependent upon whether they act at the (usually non-reducing) end or in the middle, respectively, of an oligo/polysaccharide chain. Glycoside hydrolases may also be classified by sequence based methods.

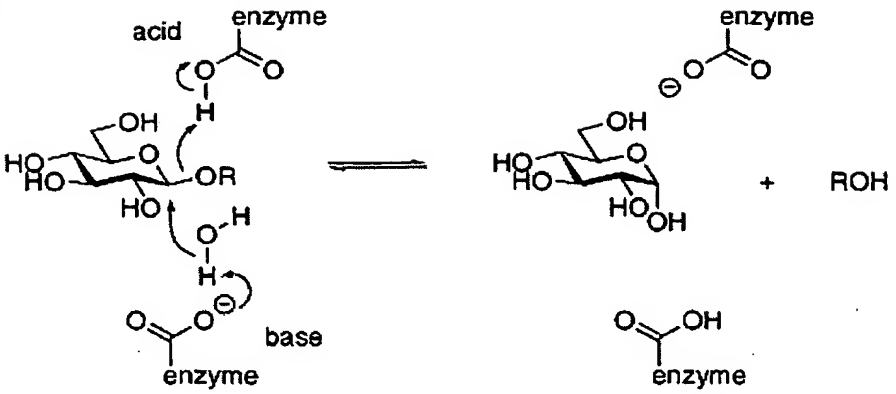
Sequence-based classification

Sequence-based classifications are among the most powerful predictive method for suggesting function for newly sequenced enzymes for which function has not been biochemically demonstrated. The sequence based classification of Henrissat and colleagues, available through the online database carbohydrate active enzymes (CAZy) provides a series of regularly updated sequence based classification that allow reliable prediction of mechanism (retaining/inverting), active site residues and possible substrates.[2]

Mechanism

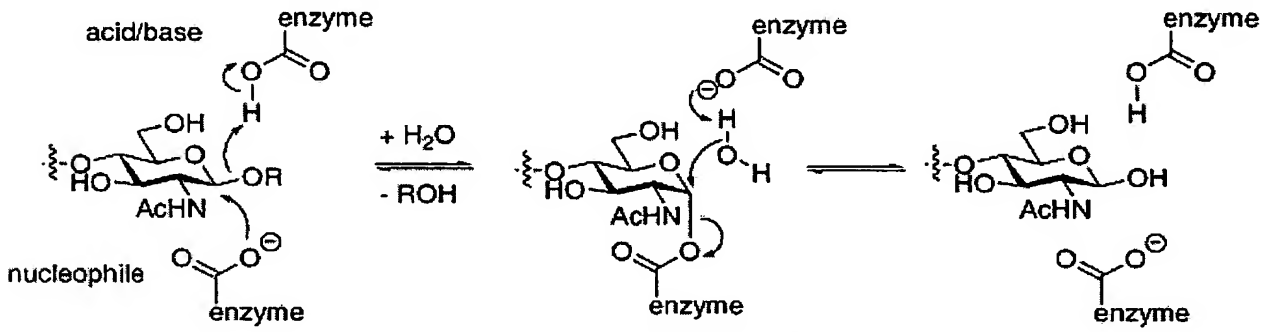
Inverting glycoside hydrolases

Inverting enzymes utilize two enzymic residues, typically carboxylate residues, that act as acid and base respectively, as shown below for a *beta*-glucosidase.



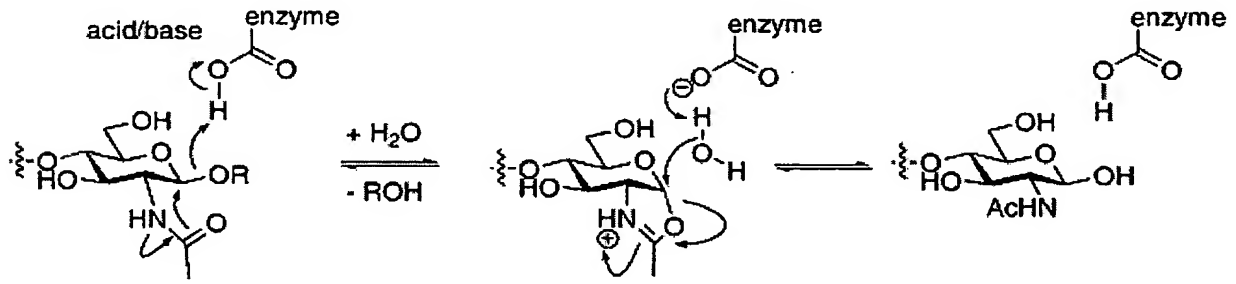
Retaining glycoside hydrolases

Retaining glycosidases operate through a two-step mechanism, with each step resulting in inversion, for a net retention of stereochemistry. Again, two residues are involved, which are usually enzyme-borne carboxylates. One acts as a nucleophile and the other as an acid/base. In the first step the nucleophile attacks the anomeric centre, resulting in the formation of a glycosyl enzyme intermediate, with acidic assistance provided by the acidic carboxylate. In the second step the now deprotoned acidic carboxylate acts as a base and assists a nucleophilic water to hydrolyze the glycosyl enzyme intermediate, giving the hydrolyzed product. The mechanism is illustrated below for hen egg white lysozyme.[3]



glycosyl enzyme intermediate

An alternative mechanism for hydrolysis with retention of stereochemistry can occur that proceeds through a nucleophilic residue that is bound to the substrate, rather than being attached to the enzyme. Such mechanisms are common for certain N-acetylhexoamindases, which have an acetamido group capable of neighboring group participation to form an intermediate oxazoline or oxazolinium ion. Again, the mechanism proceeds in two steps through individual inversions to lead to a nett retention of configuration.



oxazolinium intermediate

Nomenclature and examples

Glycoside hydrolases are typically named after the substrate that they act upon. Thus glucosidases catalyze the hydrolysis of glucosidases and xylanases catalyze the cleavage of the xylose based homopolymer xylan. Other examples include lactase, amylase, chitinase, sucrase, maltase, neuraminidase, invertase, hyaluronidase and lysozyme.

Uses

Glycoside hydrolases have a variety of uses including degradation of plant materials (eg cellulases for degrading cellulose to glucose, which can be used for ethanol production), in the food industry (invertase for use in making soft-centered chocolates), and in the paper industry (xylanases for removing hemicelluloses from paper pulp).

In organic chemistry, glycoside hydrolases can be used as synthetic catalysts to form glycosidic bonds through either reverse hydrolysis (kinetic approach) where the equilibrium position is reversed; or by transglycosylation (kinetic approach) whereby retaining glycoside hydrolases can catalyze the transfer of a glycosyl moiety from an activated glycoside to an acceptor alcohol to afford a new glycoside.

Mutant glycoside hydrolases termed glycosynthases have been developed that can achieve the synthesis of glycosides in high yield from activated glycosyl donors such as glycosyl fluorides.

See also

- Mucopolysaccharidoses
- glucosidase
- lysozyme
- Glycosyltransferase

References

1. ^ Sinnott, M. L. *Chem. Rev.* 1990, **90**, 1171
2. ^ http://afmb.cnrs-mrs.fr/CAZY/
3. ^ Vocadlo, D. J.; Davies, G. J.; Laine, R.; Withers, S. G. *Nature* 2001, **412**, 835.

Enzymes [hide]

Active site - Binding site - Catalytically perfect enzyme - Coenzyme - Cofactor - EC number - Enzyme catalysis - Enzyme kinetics - Enzyme inhibitor - Lineweaver-Burk plot - Michaelis-Menten kinetics

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EC1 Oxidoreductases/list (catalase, dehydrogenase, hydroxylase, oxygenase, oxidase, peroxidase) - **EC2 Transferases**/list (DNA methyltransferase, phosphotransferase, polymerase, DNA, I, II, III, RNA, I, II, III, primase, kinase) - **EC3 Hydrolases**/list (nuclease, esterase, phosphodiesterase, lipase, protease, DNA glycosylases, **glycosidase**, peptidase, phosphatase, phospholipase, ribonuclease) - **EC4 Lyases**/list (aldolase, carboxy-lyases, dehydratase, synthase) - **EC5 Isomerases**/list (topoisomerase, gyrase, topoisomerase IV, mutase) - **EC6 Ligases**/list (aminoacyl tRNA synthetase)

Categories: Organic chemistry | Biochemistry | EC 3.2

1: Retrovirology. 2006 Nov 8;3:80. Related Articles, Links

Human Immunodeficiency Virus-Type 1 LTR DNA contains an intrinsic gene producing antisense RNA and protein products.

Ludwig LB, Ambrus JL Jr, Krawczyk KA, Sharma S, Brooks S, Hsiao CB, Schwartz SA.

Division of Allergy, Immunology and Rheumatology, Department of Medicine, School of Biomedical Science and Medicine, State University of New York at Buffalo, Buffalo, New York 14203, USA. lbludwig@comcast.net.

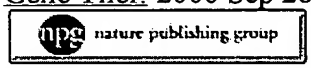
BACKGROUND: While viruses have long been shown to capitalize on their limited genomic size by utilizing both strands of DNA or complementary DNA/RNA intermediates to code for viral proteins, it has been assumed that human retroviruses have all their major proteins translated only from the plus or sense strand of RNA, despite their requirement for a dsDNA proviral intermediate. Several studies, however, have suggested the presence of antisense transcription for both HIV-1 and HTLV-1. More recently an antisense transcript responsible for the HTLV-1 bZIP factor (HBZ) protein has been described. In this study we investigated the possibility of an antisense gene contained within the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). RESULTS: Inspection of published sequences revealed a potential transcription initiator element (INR) situated downstream of, and in reverse orientation to, the usual HIV-1 promoter and transcription start site. This antisense initiator (HIVaINR) suggested the possibility of an antisense gene responsible for RNA and protein production. We show that antisense transcripts are generated, in vitro and in vivo, originating from the TAR DNA of the HIV-1 LTR. To test the possibility that protein(s) could be translated from this novel HIV-1 antisense RNA, recombinant HIV antisense gene-FLAG vectors were designed. Recombinant protein(s) were produced and isolated utilizing carboxy-terminal FLAG epitope (DYKDDDDK) sequences. In addition, affinity-purified antisera to an internal peptide derived from the HIV antisense protein (HAP) sequences identified HAPs from HIV+ human peripheral blood lymphocytes. CONCLUSION: HIV-1 contains an antisense gene in the U3-R regions of the LTR responsible for both an antisense RNA

transcript and proteins. This antisense transcript has tremendous potential for intrinsic RNA regulation because of its overlap with the beginning of all HIV-1 sense RNA transcripts by 25 nucleotides. The novel HAPs are encoded in a region of the LTR that has already been shown to be deleted in some HIV-infected long-term survivors and represent new potential targets for vaccine development.

Publication Types:
- Research Support, N.I.H., Extramural

PMID: 17090330 [PubMed - indexed for MEDLINE]

☐ **2:** Gene Ther. 2006 Sep 28; [Epub ahead of print] Related Articles, Links



Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon.

Kurachi S, Koizumi N, Sakurai F, Kawabata K, Sakurai H, Nakagawa S, Hayakawa T, Mizuguchi H.

[1] 1Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan [2] 2Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan.

Adenovirus (Ad) vectors are widely used in gene therapy and in vitro/in vivo gene transfer because of their high transduction efficiency. However, Ad vector application in the gene therapy field is limited by poor transduction into cells not expressing the primary receptor, coxsackievirus and adenovirus receptor. To overcome this problem, several types of capsid-modified Ad vectors have been developed. The HI loop or C-terminus of the fiber knob, the C-terminus of the protein IX (pIX) and the hypervariable region 5 of the hexon are promising candidate locations for displaying foreign peptide sequences. In the present study, we constructed Ad vectors in which each of the above region was modified by a simple in vitro ligation-based method, and examined the characterization of each Ad vector containing the FLAG tag (DYKDDDDK) or RGD (CDCRGDCFC) peptide. Enzyme-linked immunosorbent assay examining the surface expression of foreign peptides on the virus suggested that foreign peptides are exposed on virion surfaces in all types vectors and that the hexon was the most efficiently reacted, reflecting the copy number of the modification. However, in the case of the transduction efficiency of Ad vectors containing the RGD peptides, the modification of pIX and the hexon showed no effect. The modification of the HI loop of the fiber knob was the most efficient, followed by the modification of the C-terminus region of the fiber knob. These comparative analyses, together with a simple construction method for each modified Ad vector, could provide basic information for the generation of capsid-modified Ad vectors.Gene Therapy advance online publication, 28 September 2006; doi:10.1038/sj.gt.3302859.

PMID: 17006548 [PubMed - as supplied by publisher]

☐3: Biotechnol Prog. 2006 Jul-Aug;22(4):1170-8. Related Articles, Links

Design and characterization of stimuli-responsive FLAG-tag analogues and the illumination-induced modulation of their interaction with antibody 4E11.

Pohner C, Hilbrig F, Jerome V, Freitag R.

Chair for Process Biotechnology, University of Bayreuth, Germany.

An azobenzene group containing beta-amino acid N-Fmoc-4-aminomethyl phenylazobenzoic acid was synthesized and with the exception of the C-terminal amino acid residue was substituted by solid-phase peptide synthesis into all positions of the FLAG sequence (DYKDDDDK), an octapeptide capable of specific interaction with the monoclonal antibody 4E11. The trans state of the beta-amino acid was thermodynamically more stable than the cis state. However, the molecule could be switched into the cis conformation by illumination at 340 nm. Peptides containing the artificial amino acid also became photoresponsive. In the absence of light, the spontaneous back-isomerization into the trans conformation of the photoresponsive was extremely slow (>8 h no significant increase in trans content). When illuminated with visible light (440 nm), the back-isomerization from the cis to the trans state was accelerated and occurred with a half-life of approximately 10 min. The cis form of the photopeptides was more hydrophilic than the trans form, as evidenced by differences in the retention time of the two isomeric forms in reversed-phase chromatography. Photopeptides that contained the intact sequences responsible for binding of the FLAG tag to the antibody, namely, the DYK motive at the N-terminus, showed binding to the antibody in both a dot blot immunoassay and in Biacore binding studies, albeit with lower affinity than the unmodified FLAG sequence. Peptides with a substitution in positions 4-6 showed differences in binding strength between the trans and the cis form in the Biacore studies, no such difference could be observed for the peptide with a substitution in position 7.

PMID: 16889395 [PubMed - in process]

☐4: J Pharmacol Exp Ther. 2006 Nov;319(2):765-75. Epub 2006 Aug 1. Related Articles, Links

Ligands regulate cell surface level of the human kappa opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement:

differential effects of nonpeptide and peptide agonists.

Chen Y, Chen C, Wang Y, Liu-Chen LY.

Department of Pharmacology, Temple University School of Medicine, 3420 N. Broad St., Philadelphia, PA 19140, USA.

Two peptide agonists, eight nonpeptide agonists, and five nonpeptide antagonists were evaluated for their capacity to regulate FLAG (DYKDDDDK)-tagged human kappa opioid receptors (hKORs) stably expressed in Chinese hamster ovary cells after incubation for 4 h with a ligand at a concentration approximately 1000-fold of its EC(50) (agonist) or K(i) (antagonist) value. Dynorphins A and B decreased the fully glycosylated mature form (55-kDa) of FLAG-hKOR by 70%, whereas nonpeptide full agonists [2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl-]acetamide (U50,488H), 17-cyclopropylmethyl-3,14-dihydroxy-4,5-epoxy-6-[N-methyl-trans-3-(3-furyl) acrylamido] morphinan hydrochloride (TRK-820), ethylketocyclazocine, bremazocine, asimadoline, and (RS)-[3-[1-[[(3,4-dichlorophenyl)acetyl]-methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy] acetic acid hydrochloride (ICI 204,448) caused 10-30% decreases. In contrast, pentazocine (partial agonist) and etorphine (full agonist) up-regulated by approximately 15 and 25%, respectively. The antagonists naloxone and norbinaltorphimine also significantly increased the 55-kDa receptor, whereas selective mu, delta, and D(1) receptor antagonists had no effect. Naloxone up-regulated the receptor concentration- and time-dependently and enhanced the receptor maturation extent, without affecting its turnover. Treatment with brefeldin A (BFA), which disrupts Golgi, resulted in generation of a 51-kDa form that resided intracellularly. Naloxone up-regulated the new species, indicating that its action site is in the endoplasmic reticulum as a pharmacological chaperone. After treatment with BFA, all nonpeptide agonists up-regulated the 51-kDa form, whereas dynorphins A and B did not, indicating that nonpeptide agonists act as pharmacological chaperones, but peptide agonists do not. BFA treatment enhanced down-regulation of the cell surface receptor induced by nonpeptide agonists, but not that by peptide agonists, and unmasked etorphine- and pentazocine-mediated receptor down-regulation. These results demonstrate that ligands have dual effects on receptor levels: enhancement by chaperone-like effects and agonist-promoted down-regulation, and the net effect reflects the algebraic sum of the two.

Publication Types:
- Research Support, N.I.H., Extramural

PMID: 16882876 [PubMed - indexed for MEDLINE]

☐ **5:** Biochem J. 2006 Jun 1;396(2):347-54. Related Articles, Links

BIOCHEMICAL JOURNAL free full text article in PubMed Central

Recombinant human hyaluronan synthase 3 is phosphorylated in mammalian cells.

Goentzel BJ, Weigel PH, Steinberg RA.

Department of Biochemistry and Molecular Biology, the University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA.

Hyaluronan is a ubiquitous component of vertebrate extracellular and cell-associated matrices that serves as a key structural component of skin, cartilage, eyes and joints, and plays important roles in dynamic cellular processes, including embryogenesis, inflammation, wound healing and metastasis. Hyaluronan is synthesized by three homologous hyaluronan synthases designated HAS1, HAS2 and HAS3 that differ in their tissue distribution, regulation and enzymatic characteristics. Some progress has been made in characterizing regulation of HAS transcripts and in distinguishing the enzymatic properties of the various HAS isoforms, but essentially nothing is known about their possible regulation by posttranslational modification. Using [32P]P(i) radiolabelling of a recombinant FLAG (DYKDDDDK) epitope-tagged version of human HAS3 expressed in COS-7 cells, we show that HAS3 is serine-phosphorylated and that this phosphorylation can be enhanced by a number of effectors--most significantly by a membrane-permeable analogue of cAMP. By employing a novel FLAG-tagged phosphorylated reference protein derived from EGFP (enhanced green fluorescent protein), we were able to estimate the stoichiometry of FLAG-HAS3 phosphorylation. It was approx. 0.11 in unstimulated cells and increased to as much as 0.32 in cells stimulated with 8-(4-chlorophenylthio)-cAMP.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 16522194 [PubMed - indexed for MEDLINE]

☐ **6:** J Pharmacol Exp Ther. 2006 Jun;317(3):1295-306. Epub 2006 Feb 27. Related Articles, Links

Full Text J Pharma Exp Ther

Localization of the kappa opioid receptor in lipid rafts.

Xu W, Yoon SI, Huang P, Wang Y, Chen C, Chong PL, Liu-Chen LY.

Department of Pharmacology, Center for Substance Abuse Research, Temple University School of Medicine, 3420 N. Broad St., Philadelphia, PA 19140, USA.

Lipid rafts are microdomains of plasma membranes enriched in cholesterol and sphingolipids in the outer layer. We determined whether kappa opioid receptors (KOR) in human placenta and FLAG (DYKDDDDK)-tagged human KOR (FLAG-hKOR) expressed in Chinese hamster ovary (CHO) cells are localized in lipid rafts and

whether changes in cholesterol contents affect hKOR properties and signaling. Lipid rafts were prepared from placenta membranes and CHO cells expressing FLAG-hKOR using the Na2CO3 method and fractionation through a sucrose density gradient. The majority of the KOR in the placenta and FLAG-hKOR in CHO cells, determined by [3H]diprenorphine binding and/or immunoblotting with an anti-FLAG antibody, was present in low-density fractions, coinciding with high levels of caveolin-1 and cholesterol, markers of lipid rafts, which indicated that the KOR is localized in lipid rafts. Pretreatment with 2% methyl beta-cyclodextrin (MCD) reduced cholesterol content by approximately 48% and changed the cells from spindle-shaped to spherical. MCD treatment disrupted lipid rafts, shifted caveolin-1 and FLAG-hKOR to higher density fractions, increased the affinity of (-)-(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide (U50,488H) for the hKOR, and greatly increased U50,488H-induced [35S]guanosine 5'-O-(3-thio)triphosphate binding and p42/44 mitogen-activated protein kinase phosphorylation. Cholesterol replenishment reversed all the MCD effects. Caveolin-1 immunoprecipitated with Galphai proteins and MCD treatment reduced caveolin-1 associated with Galphai proteins, which may contribute to the enhanced agonist-induced G protein activation. Caveolin-1 also immunoprecipitated with FLAG-hKOR, but MCD treatment had no effect on the association. Thus, the KOR is located in lipid rafts and its localization in the microdomains greatly affects coupling to G proteins.

Publication Types:
- Research Support, N.I.H., Extramural
- Research Support, Non-U.S. Gov't

PMID: 16505160 [PubMed - indexed for MEDLINE]

☐ 7: Immunol Cell Biol. 2005 Oct;83(5):542-8. Related Articles, Links

nature publishing group

Production and purification of human indoleamine 2,3-dioxygenase (HuIDO) protein in a baculovirus expression system and production and characterization of egg yolk antibody against the purified HuIDO.

Webster NL, Wee J, Uren SJ, Boyle W, Sandrin MS.

Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia.

The human indoleamine 2,3-dioxygenase (HuIDO) baculoviral construct, for expression of HuIDO protein with a hexa-histidine and FLAG (DYKDDDDK) tag, was produced using the BacPAK Baculovirus Expression System. HuIDO baculovirus was used to infect Sf21 insect cells to produce functionally active protein in large amounts. Conditions for protein purification by metal affinity chromatography were determined and optimized. Addition of haemin ensured optimal activity of the purified heme-containing oxygenase. The soluble purified

protein was used to immunize a chicken to produce large quantities of polyclonal IgY against HuIDO. The anti-HuIDO IgY antibody specifically detected HuIDO produced by a range of cell types including transfectants and native HuIDO expression induced in IFN-gamma-stimulated cells. The antibody detected HuIDO in cell lysates by western blotting and in the cytoplasm of cells by microscopy. The antibody was unable to block the function of the enzyme, indicating that this antibody binds outside the active site of HuIDO.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 16174105 [PubMed - in process]

☐ **8:** J Chromatogr A. 2003 Aug 15;1009(1-2):81-7. Related Articles, Links

Complex formation of a calcium-dependent antibody: a thermodynamical consideration.

Einhauer A, **Jungbauer A**.

Institute for Applied Microbiology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria.

The elution of FLAG-fusions (an octapeptide with the sequence DYKDDDDK) from immobilized anti-FLAG antibody M1 cannot be explained by a switch of the equilibrium binding constant to a lower value. To get a further insight into thermodynamics, the binding of anti-FLAG antibody M1 to the FLAG peptide was studied by real-time biosensor technology at seven different temperatures in the range from 5 to 35 degrees C. Binding studies were performed in the presence and absence of calcium. Thermodynamic parameters such as change in Gibbs free energy (deltaG), enthalpy (deltaH) and entropy (deltaS) were evaluated from the corresponding equilibrium data applying the integrated Van't Hoff equation. In contrast to similar kinetic data obtained, the contribution of deltaH and deltaS to deltaG in the presence or absence of calcium results in a different conformation of the antibody-antigen complex under binding and non-binding conditions. Therefore, complex dissociation with EDTA must be effected during a transition state of complex formation and dissociation.

PMID: 13677647 [PubMed - indexed for MEDLINE]

☐ **9:** Biotechniques. 2002 Jun;32(6):1270, 1272-6, 1278, 1280. Related Articles, Links

Minimal FLAG sequence useful in the functional epitope tagging of H-Ras.

Johnson KY, **Liu L**, **Vincent TS**.

Medical University of South Carolina, Charleston 29425, USA.

Epitope tagging can interfere with normal protein function, indicating the need for an unobtrusive epitope tag. The FLAG epitope (DYKDDDDK) was examined for a minimal epitope useful in the tagging of H-Ras. The heptapeptide tag, F7 (MDYKDDD), was found to retain reactivity with M2 and M5 monoclonal antibodies in immunoprecipitation, Western blotting, and immunofluorescence microscopy. The F7 tag did not interfere with Ras stability, EGF stimulation of Ras activation, and downstream phosphorylation of MAPK Erk1/2. Unlike the full FLAG sequence, the F7 tag had minimal effect on the growth properties of H-Ras in a colony-forming assay. The F7 tag may be useful when minimizing the effect of tagging on protein function is an important criterion in the selection of an N-terminal epitope tag.

PMID: 12074157 [PubMed - indexed for MEDLINE]

☐ **10:** DNA Cell Biol. 2002 Mar;21(3):151-62. Related Articles, Links

Mary Ann Liebert,

Human herpesvirus-8 encoded Kaposin: subcellular localization using immunofluorescence and biochemical approaches.

Tomkowicz B, Singh SP, Cartas M, Srinivasan A.

Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA.

Human herpesvirus-8 (HHV-8) has been causally linked to the development of Kaposi's sarcoma (KS). DNA sequence analysis of the viral genome revealed a total of 81 open reading frames (ORF). Interestingly, only a small subset of these ORFs has been shown to be transcribed in cells latently infected with HHV-8 and in cells of the KS lesions. Among the genes active during latency, kaposin, is noted for its abundance and ability to transform cells in culture, thus implicating a potential role in KS pathogenesis. This has prompted us to undertake an investigation on elucidating the mechanism(s) by which Kaposin brings about transformation of cells. Towards this goal, we have generated an eukaryotic expression plasmid encoding Kaposin (Kap). As Kaposin is predicted to be a type II membrane protein, several strategies were utilized to address this, including the generation of Kaposin with the Flag (FL) epitope (DYKDDDDK) at the C-terminus of the protein (Kap-C-FL). Antibodies specific for Kaposin (kap-2), recognized both Kaposin and Kaposin-Flag, while antibodies against the Flag epitope recognized only Kaposin-Flag. Transfection of Kap and Kap-C-FL expression plasmid DNA into NIH3T3 cells resulted in cellular clones that exhibited a phenotypic property of transformation by forming large, multiclustered cells, when grown on soft agar. Because there is controversial data regarding the localization of Kaposin in cells, we examined the subcellular localization of Kaposin using confocal

microscopy. We observed that Kaposin and Kaposin-Flag showed an intense staining surrounding the nucleus. Although there was no staining at the cell membrane of transfected cells, FACS analysis using kap-2 or Flag antibodies, under nonpermeable conditions, showed positivity. Cell fractionation studies further showed that the majority of Kaposin was detected in the nuclear fraction by Western blot analysis. The cytoplasmic and detergent soluble membrane fractions did not show Kaposin protein; however, a small amount was detected in the detergent insoluble membrane fraction. Taken together, these results suggest that Kaposin exhibits multicompartmental localization in cells.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 12015894 [PubMed - indexed for MEDLINE]

☐ **11:** Pflugers Arch. 2001 Nov;443(2):280-8. Related Articles, Links

SpringerLink FULL-TEXT ARTICLE

Topology of the human skeletal muscle chloride channel hClC-1 probed with hydrophilic epitope insertion.

Kuchenbecker M, **Schu B**, **Kurz L**, **Rudel R**.

Department of General Physiology, University of Ulm, 89060 Ulm, Germany.

To investigate the membrane topology of the skeletal muscle chloride channel ClC-1, we inserted the small antigenic flag (DYKDDDDK) and/or HSV (QPELAPEDPED) epitope tags into nine predicted extra- and intracellular loops along the channel protein. Functional integrity of the modified proteins was tested by measuring the chloride currents conducted by these channels expressed in tsA201 cells. Insertion of the tags into the linkers D1D2, D4D5, D6D7, D8D9 or D11D12 did not alter channel function significantly, whereas insertion into D3D4, D5D6, D9D10 and D10D11 led to loss of function. Intra- or extracellular localisation of the tags was determined by immunofluorescent staining of intact and permeabilised tsA201 cells transiently transfected with the functional epitope-inserted constructs. Intact cells stained for the epitope tags inserted into D1D2, D6D7 and D8D9, indicating that these linkers face the extracellular side of the membrane. No conclusions could be drawn for the location of D4D5 and D11D12. Insertion of the flag epitope at position P260 (linker D4D5), a putative pore-lining region, did not change any of the channel function properties markedly, suggesting that the region surrounding P260 cannot directly line the ion conduction pathway of ClC-1.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 11713655 [PubMed - indexed for MEDLINE]

☐ **12:** J Biol Chem. 2001 Jul 27;276(30):27881-92. Epub 2001 May 3. Related Articles, Links

Addition of a glycophosphatidylinositol to acetylcholinesterase. Processing, degradation, and secretion.

Coussen F, Ayon A, Le Goff A, Leroy J, Massoulie J, Bon S.

Laboratoire de Neurobiologie Moleculaire et Cellulaire, CNRS UMR 8544, Ecole Normale Superieure, 46 rue d'Ulm, 75005 Paris, France.

We introduced various mutations and modifications in the GPI anchoring signal of rat acetylcholinesterase (AChE). 1) The resulting mutants, expressed in transiently transfected COS cells, were initially produced at the same rate, in an active form, but the fraction of GPI-anchored AChE and the steady state level of AChE activity varied over a wide range. 2) Productive interaction with the GPI addition machinery led to GPI anchoring, secretion of uncleaved protein, and secretion of a cleaved protein, in variable proportions. Unproductive interaction led to degradation; poorly processed molecules were degraded rather than retained intracellularly or secreted. 3) An efficient glypiation appeared necessary but not sufficient for a high level of secretion; the cleaved, secreted protein was possibly generated as a by-product of transamidation. 4) Glypiation was influenced by a wider context than the triplet omega/omega + 1/omega + 2, particularly omega - 1. 5) Glypiation was not affected by the closeness of the omega site to the alpha(10) helix of the catalytic domain. 6) A cysteine could simultaneously form a disulfide bond and serve as an omega site; however, there was a mutual interference between glypiation and the formation of an intercatenary disulfide bond, at a short distance upstream of omega. 7) Glypiation was not affected by the presence of an N-glycosylation site at omega or in its vicinity or by the addition of a short hydrophilic, highly charged peptide (FLAG; DYKDDDDK) at the C terminus of the hydrophobic region.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 11337488 [PubMed - indexed for MEDLINE]

☐ **13:** Biochim Biophys Acta. 2001 Mar 9;1511(1):80-9. Related Articles, Links

Topology of the Na(+)/dicarboxylate cotransporter: the N-terminus and hydrophilic loop 4 are located intracellularly.

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The current secondary structure model of the Na(+)/dicarboxylate cotransporter, NaDC-1, contains 11 transmembrane domains. The model is based on hydropathy analysis and the extracellular location of the carboxy terminus, which contains an N-glycosylation site. In this study, the model was further tested using indirect immunofluorescence of COS-7 cells. The Flag epitope tag (DYKDDDDK) was fused to the amino terminus of NaDC-1 (Flag-NaDC-1), and a monoclonal antibody against the Flag epitope was used to determine the location of the N-terminus. Hydrophilic loop 4 of NaDC-1 was identified using polyclonal antibodies raised against a fusion protein containing amino acids 164--233 of NaDC-1. The expression of NaDC-1 and Flag-NaDC-1 in COS-7 cells was confirmed by functional assays of succinate transport and by Western blots of cell surface biotinylated proteins. Immunofluorescent labeling of cells expressing both NaDC-1 and Flag-NaDC-1 required permeabilization of the plasma membranes with digitonin whereas no immunofluorescence was visible in intact cells. The results of this study show that both the N-terminus and hydrophilic loop 4 of NaDC-1 are located intracellularly, which supports the current model of NaDC-1 structure.

Publication Types:
- Research Support, U.S. Gov't, P.H.S.

PMID: 11248207 [PubMed - indexed for MEDLINE]

☐ **14:** J Neuroendocrinol. 2001 Jan;13(1):94-101. Related Articles, Links

Full Text at Blackwell Synergy

Characterization of an antibody to the human melatonin mt1 receptor.

Williams LM, Drew JE, Bunnett NW, Grady E, Barrett P, Abramovich DR, Morris A, Slater D.

Molecular Physiology Group, The Rowett Research Institute, Aberdeen, UK. l.williams@rri.sari.ac.uk

Melatonin acts via high affinity, G-protein coupled, seven transmembrane domain receptors. To precisely localize these receptors, antibodies were raised in chickens against a 15 amino acid fragment at the intracellular C-terminal region of the human melatonin receptor subtype mt1 (DSSNDVADRVKWKPS, mt(1338-352)). A chimeric form of the receptor with a hydrophilic Flag peptide (DYKDDDDK) in sequence with the extracellular N-terminus (Flag-mt1) was generated by polymerase chain reaction and expressed in mammalian cell lines. An IgY antibody (Y31), which gave high antibody titres by enzyme-linked immunosorbent assay, was used to localize Flag-mt1 in stably transfected cells by immunofluoresence. Flag-mt1 localization with Y31 was identical to that obtained with the M5 antibody directed against the Flag epitope and was mainly localized to the Golgi apparatus with some

staining at the cell surface. No staining was seen in untransfected cells with either antibody. Y31 staining was abolished using antibody preabsorbed with peptide antigen. Y31 immunofluorescence in fetal human kidney sections was restricted to nephrogenic regions and matched that of 2-((125)I)iodomelatonin binding and mt1 gene expression by in situ hybridization. Y31 was used to immunoprecipitate biotinylated membrane proteins from Flag-mt1 stably transfected and untransfected CHO cells. Western blotting of immunoprecipitated proteins revealed two major bands specific to stably transfected cells, one at 63 kDa and one at 86 kDa. The first band almost certainly corresponds to the glycosylated form of Flag-mt1 and the second band to receptor dimers. Thus, Y31 antibody is suitable for use in detecting the human mt1 receptor subtype in tissues and in transfected cells.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 11123519 [PubMed - indexed for MEDLINE]

☐ **15:** J Virol. 2000 Nov;74(22):10650-7. Related Articles, Links

Functional role of residues corresponding to helical domain II (amino acids 35 to 46) of human immunodeficiency virus type 1 Vpr.

Singh SP, Tomkowicz B, Lai D, Cartas M, Mahalingam S, Kalyanaraman VS, Murali R, Srinivasan A.

Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA.

Vpr, encoded by the human immunodeficiency virus type 1 genome, contains 96 amino acids and is a multifunctional protein with features which include cell cycle arrest at G(2), nuclear localization, participation in transport of the preintegration complex, cation channel activity, oligomerization, and interaction with cellular proteins, in addition to its incorporation into the virus particles. Recently, structural studies based on nuclear magnetic resonance and circular dichroism spectroscopy showed that Vpr contains a helix (HI)-turn-helix (HII) core at the amino terminus and an amphipathic helix (HIII) in the middle region. Though the importance of helical domains HI and HIII has been defined with respect to Vpr functions, the role of helical domain HII is not known. To address this issue, we constructed a series of mutants in which the HII domain was altered by deletion, insertion, and/or substitution mutagenesis. To enable the detection of Vpr, the sequence corresponding to the Flag epitope (DYKDDDDK) was added, in frame, to the Vpr coding sequences. Mutants, expressed through the in vitro transcription/translation system and in cells, showed an altered migration corresponding to deletions in Vpr. Substitution mutational analysis of residues in HII showed reduced stability for VprW38S-FL, VprL42G-FL, and VprH45W-FL. An assay involving cotransfection of

NLDeltaVpr proviral DNA and a Vpr expression plasmid was employed to analyze the virion incorporation property of Vpr. Mutant Vpr containing deletions and specific substitutions (VprW38S-FL, VprL39G-FL, VprL42G-FL, VprG43P-FL, and VprI46G-FL) exhibited a negative virion incorporation phenotype. Further, mutant Vpr-FL containing deletions also failed to associate with wild-type Vpr, indicating a possible defect in the oligomerization feature of Vpr. Subcellular localization studies indicated that mutants VprDelta35-50-H-FL, VprR36W-FL, VprL39G-FL, and VprI46G-FL exhibited both cytoplasmic and nuclear localization, unlike other mutants and control Vpr-FL. While wild-type Vpr registered cell cycle arrest at G(2), mutant Vpr showed an intermediary effect with the exception of VprDelta35-50 and VprDelta35-50-H. These results suggest that residues in the HII domain are essential for Vpr functions.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 11044109 [PubMed - indexed for MEDLINE]

☐ **16:** Toxicon. 2001 Feb-Mar;39(2-3):291-301. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Expression and characterization of Flag-epitope- and hexahistidine-tagged derivatives of saxiphilin for use in detection and assay of saxitoxin.

Krishnan G, Morabito MA, Moczydlowski E.

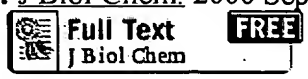
Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8066, USA.

Saxiphilin is a plasma protein from the bullfrog (Rana catesbiana) that binds saxitoxin (STX), a causative agent of paralytic shellfish poisoning. Saxiphilin is homologous to transferrin and consists of two internally homologous domains called the N-lobe and the C-lobe. STX binds to a single site in the C-lobe of saxiphilin. In this study, cloned genes coding for recombinant saxiphilin and C-lobe saxiphilin were modified to contain two tandemly located affinity tags, Flag epitope (DYKDDDDK) and His(6) (HHHHHH), at the protein C-terminus and were expressed in cultured insect cells using baculovirus vectors. Both tagged proteins are readily detected on immunoblots by anti-Flag monoclonal antibody. Flag-His(6)-tagged saxiphilin was purified to homogeneity using Ni(2+)-chelate affinity chromatography and Heparin Sepharose chromatography. Equilibrium analysis of [3H]STX binding to tagged saxiphilin and tagged C-lobe saxiphilin gave K(D) values of 0.75 and 2.7 nM, respectively. Flag-His(6)-tagged saxiphilin was also utilized in a microtiter well solid-phase assay with Reacti-bind metal chelate plates to measure [3H]STX binding and binding competition by unlabeled STX. Such Flag-His(6)-tagged derivatives of saxiphilin have many possible applications in the assay of STX and related toxinological research.

Publication Types:
- Research Support, U.S. Gov't, P.H.S.

PMID: 10978747 [PubMed - indexed for MEDLINE]

☐ 17: J Biol Chem. 2000 Sep 22;275(38):29324-30. Related Articles, Links



High affinity binding between lipoprotein lipase and lipoproteins involves multiple ionic and hydrophobic interactions, does not require enzyme activity, and is modulated by glycosaminoglycans.

Hussain MM, Obunike JC, Shaheen A, Hussain MJ, Shelness GS, Goldberg IJ.

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Lipoprotein lipase (LPL) physically associates with lipoproteins and hydrolyzes triglycerides. To characterize the binding of LPL to lipoproteins, we studied the binding of low density lipoproteins (LDL), apolipoprotein (apo) B17, and various apoB-FLAG (DYKDDDDK octapeptide) chimeras to purified LPL. LDL bound to LPL with high affinity (K(d) values of 10(-12) m) similar to that observed for the binding of LDL to its receptors and 1D1, a monoclonal antibody to LDL, and was greater than its affinity for microsomal triglyceride transfer protein. LDL-LPL binding was sensitive to both salt and detergents, indicating the involvement of both hydrophobic and hydrophilic interactions. In contrast, the N-terminal 17% of apoB interacted with LPL mainly via ionic interactions. Binding of various apoB fusion peptides suggested that LPL bound to apoB at multiple sites within apoB17. Tetrahydrolipstatin, a potent enzyme activity inhibitor, had no effect on apoB-LPL binding, indicating that the enzyme activity was not required for apoB binding. LDL-LPL binding was inhibited by monoclonal antibodies that recognize amino acids 380-410 in the C-terminal region of LPL, a region also shown to interact with heparin and LDL receptor-related protein. The LDL-LPL binding was also inhibited by glycosaminoglycans (GAGs); heparin inhibited the interactions by approximately 50% and removal of trace amounts of heparin from LPL preparations increased LDL binding. Thus, we conclude that the high affinity binding between LPL and lipoproteins involves multiple ionic and hydrophobic interactions, does not require enzyme activity and is modulated by GAGs. It is proposed that LPL contains a surface exposed positively charged amino acid cluster that may be important for various physiological interactions of LPL with different biologically important molecules. Moreover, we postulate that by binding to this cluster, GAGs modulate the association between LDL and LPL and the in vivo metabolism of LPL.

Publication Types:

- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 10882743 [PubMed - indexed for MEDLINE]

☐ **18:** Mol Cell Biol Res Commun. 2000 Feb;3(2):115-21. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Functional expression and biochemical characterization of an epitope-tagged connexin37.

Larson DM, **Seul KH**, **Berthoud VM**, **Lau AF**, **Sagar GD**, **Beyer EC**.

Mallory Institute of Pathology, Boston University School of Medicine, Boston, Massachusetts 02118, USA. davlars@bu.edu

To study the gap junction protein connexin37 (Cx37), we stably transfected cell lines with constructs of human Cx37 containing the epitope tag FLAG (DYKDDDDK). A Cx37 construct containing the FLAG moiety at the carboxyl terminus (Cx37F) was expressed in BWEM cells, and did not substantially alter the levels of endogenous Cx43 in these cells. Immunostaining showed that Cx37F colocalized with Cx43 at cell-cell contacts. Pulse-chase metabolic labeling and immunoprecipitation with anti-FLAG antibodies indicated that Cx37F was synthesized as a protein that ran at 35.9 +/- 0.9 kDa on reducing SDS-PAGE but chased into a slower migrating band at 38.0 +/- 1.0 kDa. This shift in mobility was due to phosphorylation on serine residues, based on [(32)P]-metabolic labeling, immunoprecipitation, and phosphoamino acid analyses. The transition to the phosphoCx37F correlated with a loss of solubility in 1% Triton X-100. Based on the [(35)S]-methionine pulse-chase experiments, the half-life of the labeled Cx37F was approximately 3 h, which is within the range reported for other connexins. Analysis of dye injection experiments indicated that dye transfer was reduced in Cx37-transfected cells in comparison to parental BWEM cells, suggesting that formation of heteromeric Cx37-Cx43 channels reduced the molecular permeability of communication between these cells. Moreover, the similarities of previously demonstrated kinetic details and modification of Cx43 to our new data regarding Cx37 provide evidence for a commonality in processing and assembly steps of these two connexins.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 10775509 [PubMed - indexed for MEDLINE]

☐ **19:** Virology. 2000 Mar 15;268(2):364-71. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Epitope-tagging approach to determine the stoichiometry of

the structural and nonstructural proteins in the virus particles: amount of Vpr in relation to Gag in HIV-1.

Singh SP, Lai D, Cartas M, Serio D, Murali R, Kalyanaraman VS, Srinivasan A.

Department of Microbiology, Kimmel Cancer Center, Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania, 19107, USA.

We used an epitope-tagging approach to determine the ratio of Gag (structural) to Vpr (nonstructural) in the virus particles directed by human immunodeficiency virus type 1. For this purpose, chimeric Gag and Vpr expression plasmids were constructed with the Flag epitope (DYKDDDDK), and the sequences corresponding to the chimeric protein were introduced into human immunodeficiency virus type 1 proviral DNA (NL4-3) to determine the ratio in the virus particles when these proteins are expressed in cis. In addition, NL4-3 DNA was modified to disrupt Vpr synthesis to determine the extent of incorporation of Vpr-FL when it is expressed in trans through a heterologous promoter. The analysis of virus particles generated by transfection of proviral DNA into RD cells indicated that (1) the ratio of Gag to Vpr in virus particles, when Vpr-FL is expressed in cis (in the context of proviral DNA), is in the range of 150-200:1 (14-18 molecules of Vpr per virion) and (2) the expression of Vpr-FL in trans showed efficient incorporation with a Gag to Vpr ratio of 5-7:1 (392-550 molecules of Vpr). These results suggest that the presence of the same epitope on different viral proteins may provide an accurate comparison of these proteins in the virus particles. Copyright 2000 Academic Press.

Publication Types:
- Research Support, U.S. Gov't, P.H.S.

PMID: 10704344 [PubMed - indexed for MEDLINE]

☐ **20:** Biochem J. 2000 Feb 15;346 Pt 1:77-82. Related Articles, Links

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Mutations of the serine phosphorylated in the protein phosphatase-1-binding motif in the skeletal muscle glycogen-targeting subunit.

Liu J, Wu J, Oliver C, Shenolikar S, Brautigan DL.

Center for Cell Signaling, University of Virginia School of Medicine, Box 577, West Complex MSB 7196, Charlottesville, VA 22908, USA.

Cellular functions of protein phosphatase-1 (PP1) are determined by regulatory subunits that contain the consensus PP1-binding motif, RVXF. This motif was first identified as the site of phosphorylation by cAMP-dependent protein kinase (PKA) in a skeletal muscle glycogen-targeting subunit (G(M)). We reported previously that a recombinant

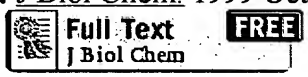
fusion protein of glutathione S-transferase (GST) and the N-terminal domain of G(M) [GST-G(M)-(1-240)] bound PP1 in a pull down assay, and phosphorylation by PKA prevented PP1 binding. Here we report that substitution of either Ala or Val for Ser-67 in the RVS(67)F motif in GST-G(M)-(1-240) essentially eliminated PP1 binding. This was unexpected because other glycogen-targeting subunits have a Val residue at the position corresponding to Ser-67. In contrast, a mutation of Ser-67 to Thr (S67T) in GST-G(M)(1-240) gave a protein that bound PP1 the same as wild type and was unaffected by PKA phosphorylation. Full length G(M) tagged with the epitope sequence DYKDDDDK (FLAG) expressed in COS7 cells bound PP1 that was recovered by co-immunoprecipitation, but this association was prevented by treatment of the cells with forskolin. By comparison, PP1 binding with FLAG-G(M) (S67T) was not disrupted by forskolin treatment. Neither FLAG-G(M) (S67A) nor FLAG-G(M)(S67V) formed stable complexes with PP1 in COS7 cells. These results emphasise the unique contribution of Ser-67 in PP1 binding to G(M). The constitutive PP1-binding activity shown by G (M)(S67T) opens the way for studying the role of G(M) multisite phosphorylation in hormonal control of glycogen metabolism.

Publication Types:

- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, Non-P.H.S.
- Research Support, U.S. Gov't, P.H.S.

PMID: 10657242 [PubMed - indexed for MEDLINE]

☐ **21:** J Biol Chem. 1999 Oct 29;274(44):31445-55. Related Articles, Links



Covalent homodimers of murine secretory component induced by epitope substitution unravel the capacity of the polymeric Ig receptor to dimerize noncovalently in the absence of IgA ligand.

Crottet P, Peitsch MC, Servis C, Corthesy B.

Institut Suisse de Recherches, Experimentales sur le Cancer, CH-1066 Epalinges, Switzerland.

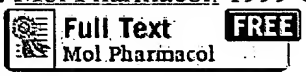
Recombinant secretory immunoglobulin A containing a bacterial epitope in domain I of the secretory component (SC) moiety can serve as a mucosal delivery vehicle triggering both mucosal and systemic responses (Corthesy, B., Kaufmann, M., Phalipon, A., Peitsch, M., Neutra, M. R., and Kraehenbuhl, J.-P. (1996) J. Biol. Chem. 271, 33670-33677). To load recombinant secretory IgA with multiple B and T epitopes and extend its biological functions, we selected, based on molecular modeling, five surface-exposed sites in domains II and III of murine SC. Loops predicted to be exposed at the surface of SC domains were replaced with the DYKDDDDK octapeptide (FLAG). Another two mutants were obtained with the FLAG inserted in between domains II and III or at the carboxyl terminus of SC. As shown by mass

spectrometry, internal substitution of the FLAG into four of the mutants induced the formation of disulfide-linked homodimers. Three of the dimers and two of the monomers from SC mutants could be affinity-purified using an antibody to the FLAG, mapping them as candidates for insertion. FLAG-induced dimerization also occurred with the polymeric immunoglobulin receptor (pIgR) and might reflect the so-far nondemonstrated capacity of the receptor to oligomerize. By co-expressing in COS-7 cells and epithelial Caco-2 cells two pIgR constructs tagged at the carboxyl terminus with hexahistidine or FLAG, we provide the strongest evidence reported to date that the pIgR dimerizes noncovalently in the plasma membrane in the absence of polymeric IgA ligand. The implication of this finding is discussed in terms of IgA transport and specific antibody response at mucosal surfaces.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 10531346 [PubMed - indexed for MEDLINE]

☐ **22:** Mol Pharmacol. 1999 Oct;56(4):705-13. Related Articles, Links



Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells.

Linden J, Thai T, Figler H, Jin X, Robeva AS.

Department of Internal Medicine, University of Virginia, Charlottesville, Virginia, USA. jlinden@virginia.edu

Recombinant human A(2B) adenosine receptors (A(2B)ARs) and receptors extended on the amino terminus with hexahistidine and the FLAG epitope, DYKDDDDK (H/F-A(2B)) were stably overexpressed (to >20,000 fmol/mg protein) in human embryonic kidney 293 cells (HEK-A(2B)). By Western blotting, the H/F-A(2B) receptor runs as a 34.8-kDa glycoprotein. Pharmacological properties of A(2B)ARs were characterized with (125)I-3-aminobenzyl-8-phenyl-(4-oxyacetic acid)-1-propylxanthine (K(D), 36 nM). In competition binding assays, the affinity of agonists is reduced by substitution on either the N(6)- or the C-2 position of the adenine ring, whereas 5'-substitutions increase affinity, resulting in the potency order: 5'-N-ethylcarboxamidoadenosine (NECA) >> N(6)-aminobenzyl-NECA approximately 2-chloroadenosine > 2-[4-(2-carboxyethyl)phenethylamino]-NECA (CGS21680) > N(6)-aminobenzyladenosine. The A(2B)AR is potently blocked by the A(2A)-selective antagonist 4-(2-[7-amino-2-[2-furyl][1,2, 4]triazolo-[2,3-a] [1,3,5] triazin-5-yl-amino]ethyl)phenol (ZM241385; K(I), 32 nM for A (2B), 1.4 nM for A(2A)) and the A(1) selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (K(I), 50.5 nM for A(2B); 2.5 nM for A(1)). The K(I) values for the antiasthmatic xanthines, theophylline (7.8 microM) and enprofylline (6.4 microM), are below their therapeutic

plasma concentrations (20 to 50 microM), and agree with K(I) determinations for inhibition of NECA-stimulated cAMP accumulation in HEK-A(2B) cells. NECA or N(6)-(2-iodo)benzyl-5'-N-methylcarboxamidodoadenosine (IB-MECA) stimulate inositol trisphosphates and calcium accumulation in HEK-A(2B) or HEK-A(3) cells, respectively, but only the A(3) response is prevented by pertussis toxin. In human HMC-1 mast cells, A(2B)AR activation stimulates calcium mobilization and cAMP accumulation. We conclude that HEK-A(2B) cells and HMC-1 mast cells possess A(2B)AR glycoproteins that are coupled to both G(q/11) and G(s).

Publication Types:
- Research Support, U.S. Gov't, P.H.S.

PMID: 10496952 [PubMed - indexed for MEDLINE]

☐ **23:** Biochem J. 1999 Aug 15;342 (Pt 1):153-61. Related Articles, Links

BIOCHEMICAL JOURNAL free full text article in PubMed Central

Biosynthesis of inositol trisphosphate receptors: selective association with the molecular chaperone calnexin.

Joseph SK, **Boehning D**, **Bokkala S**, **Watkins R**, **Widjaja J**.

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A prominent labelled polypeptide having the same mobility as type-I inositol trisphosphate receptor (IP(3)R) was immunoprecipitated from WB-cell lysates by antibodies to calnexin, an ER integral membrane chaperone. The identity of this polypeptide was confirmed by re-immunoprecipitation of the radioactive polypeptides released from calnexin-antibody immunoprecipitates with type-I IP(3)R antibody. The interaction of calnexin with newly synthesized type-I IP(3)R was transient and inhibited by treatment of the cells with dithiothreitol or the glucosidase inhibitor N-methyldeoxynojirimicin. In similar experiments, there was no evidence for the binding of type-I IP(3)R to calreticulin, an ER luminal chaperone. Calnexin (but not calreticulin) associated with newly synthesized FLAG (DYKDDDDK epitope)-tagged type-III IP(3) R expressed in COS-7 cells. In order to further define the mechanism of interaction of nascent IP(3)R with chaperones, we have utilized an in vitro rabbit reticulocyte translation assay programmed with RNA templates encoding the six putative transmembrane (TM) domains of IP (3)Rs. In accordance with the known preference of calnexin for monoglucosylated oligosaccharide chains, calnexin antibody preferentially immunoprecipitated a proportion of glycosylated type-I translation product. Addition of glucosidase inhibitors prevented the association of calnexin with in vitro translated type-I TM construct. Using truncated RNA templates we found that calnexin did not associate with the first four TM domains but retained affinity for the construct encoding TM domains 5 and 6, which contains the glycosylation sites.

We propose that calnexin is a key chaperone involved in the folding, assembly and oligomerization of newly synthesized IP(3) receptors in the ER.

Publication Types:
- Comparative Study
- Research Support, U.S. Gov't, P.H.S.

PMID: 10432312 [PubMed - indexed for MEDLINE]

☐ **24:** Growth Factors. 1999;16(4):265-78. Related Articles, Links

The N-terminus of gp130 is critical for the formation of the high-affinity interleukin-6 receptor complex.

Moritz RL, Ward LD, Tu GF, Fabri LJ, Ji H, Yasukawa K, Simpson RJ.

Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research, Melbourne, Australia.

Interleukin-6 (IL-6) mediates its activity through binding to two cell-surface receptors. The high-affinity human IL-6 receptor complex consists of two transmembrane anchored subunits: a ligand-specific, low-affinity IL-6 receptor and the high-affinity converter and signal transducing, gp130. Previously, using recombinant forms of human IL-6 and the extracellular ('soluble') domains of the IL-6 receptor (sIL-6R) and gp130 (sgp130), we have shown that the high-affinity IL-6R complex is hexameric, consisting of two molecules each of IL-6, sIL-6R and sgp130 (Ward et al., 1994, J. Biol. Chem. 269: 23286-23289). This paper investigates the role of the N-terminal region of gp130 in the formation of the high-affinity IL-6R complex. Using recombinant sgp130 produced with a FLAG octapeptide epitope (DYKDDDDK) at the N-terminus (sgp130-FLAG), we demonstrate, using biosensor analysis and size-exclusion chromatography, that modification of the N-terminus of sgp130 interferes with the in vitro in solution formation of the stable hexameric IL-6 receptor complex. Rather, sgp130-FLAG interacts with IL-6 and sIL-6R with a much lower affinity and forms a stable lower-order ternary complex. However, this lower-order complex is inconsistent with the solution molecular weight of a trimeric complex, as measured by size-exclusion chromatography. In contrast, N-terminal modification of the sgp130 with the FLAG epitope did not interfere with the binding of leukemia inhibitory factor or oncostatin-M (other cytokines that signal through gp130) to sgp130. These data support our model of the hexameric IL-6 receptor complex, which is biased towards the association of two IL-6.IL-6R.gp130 trimers, and postulates the critical involvement of the N-terminal Ig-like domain of gp130 in tethering the two trimers to form the stable hexamer (Simpson et al., 1997, Prot. Sci. 6: 929-955).

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 10427501 [PubMed - indexed for MEDLINE]

☐ **25:** Protein Expr Purif. 1998 Jun;13(1):111-9. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

The human D1A dopamine receptor: heterologous expression in Saccharomyces cerevisiae and purification of the functional receptor.

Andersen B, **Stevens RC**.

Life Sciences Division, Donner Laboratory, Lawrence Berkeley Laboratory, Berkeley, California 94720, USA.

Functional human D1A dopamine receptor has been expressed in Saccharomyces cerevisiae. The primary sequence of the receptor was modified to include two affinity tags at the C-terminus of the protein, a FLAG tag (DYKDDDDK), and a His6 tag (HHHHHH). These modifications allowed for purification to near homogeneity using immobilized metal affinity chromatography and immunoaffinity chromatography. Radioligand binding demonstrated that the purified and reconstituted receptor binds the antagonist [3H]SCH23390 with an affinity (KD = 8.0 +/- 3.2 nM) comparable to that of the native receptor.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, Non-P.H.S.
- Research Support, U.S. Gov't, P.H.S.

PMID: 9631523 [PubMed - indexed for MEDLINE]

☐ **26:** Biochem J. 1997 Nov 1;327 (Pt 3):747-57. Related Articles, Links

BIOCHEMICAL JOURNAL free full text article in PubMed Central

Tetramerization domain of human butyrylcholinesterase is at the C-terminus.

Blong RM, **Bedows E**, **Lockridge O**.

Eppley Institute and Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198-6805, USA.

Butyrylcholinesterase (BChE) in human serum consists predominantly of tetramers. Recombinant BChE, however, expressed in Chinese hamster ovary (CHO) cells, consists of approx. 55% dimers, 10-30% tetramers and 15-40% monomers. To determine the origin of the monomer species we added the FLAG epitope (epitope tag, amino acid sequence DYKDDDDK) to the C-terminus of the enzyme, and

expressed BChE-FLAG in CHO cells. We found that secreted, active monomers had lost their FLAG epitope, suggesting that the monomers were made by proteolysis of dimers or tetramers at the C-terminus. To estimate the number of amino acids that could be deleted from the C-terminus without losing BChE activity, we expressed deletion mutants. We found that deletion of up to 50 amino acids from the C-terminus yielded active monomers, but that deletion of 51 amino acids destroyed BChE activity and caused the inactive protein to remain within the cell. Deletion of eight or more amino acids from the N-terminus also resulted in inactive protein that remained inside the cell. Monomeric BChE had wild-type Km and kcat values (8 microM and 24000 min-1 for butyrylthiocholine) and showed substrate activation. The Cys-571-->Ala mutant, though incapable of forming the interchain disulphide bond, had nearly the same amount of tetrameric BChE as recombinant wild-type BChE. These results support the conclusion that the tetramerization domain of BChE is at the C-terminus, within the terminal 50 amino acids, and that the interchain disulphide bond is not essential for tetramerization. Molecular modelling suggested that the tetramerization domain was a four-helix bundle, stabilized by interactions of seven conserved aromatic amino acids.

Publication Types:
- Research Support, U.S. Gov't, Non-P.H.S.
- Research Support, U.S. Gov't, P.H.S.

PMID: 9581552 [PubMed - indexed for MEDLINE]

☐ **27:** Eur J Biochem. 1998 Jan 15;251(1-2):122-32. Related Articles, Links

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Topology of subunit a of the Escherichia coli ATP synthase.

Jager H, Birkenhager R, Stalz WD, Altendorf K, Deckers-Hebestreit G.

Universitat Osnabruck, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, Germany.

The antigenic determinants of mAbs against subunit a of the Escherichia coli ATP synthase were mapped by ELISA using overlapping synthetic decapeptides. For two of the mAbs the epitopes are E4NMTPQD10 (GDH 14-5C6) and V29DPQ32 (GDH 8-8B3). Binding of these mAbs to membrane vesicles of different orientation revealed that both epitopes are accessible in vesicles with inside-out orientation. These results demonstrate that at least the N-terminal amino acids 1-32 of subunit a are located at the cytoplasmic side of the membrane. A further determination of the topology of subunit a was performed by inserting the reporter epitope DYKDDDDK (FLAG epitope) at different positions of the polypeptide chain. 10 of 13 insertions led to a functional F0F1 ATP synthase and allowed specific detection of the modified subunit a by immunoblotting using an mAb against the FLAG epitope. In addition, polyclonal anti-FLAG IgG was applied for the recognition of the mutant

FLAG epitope DYKDDVDK. Cells carrying this mutant FLAG epitope at the C terminus of subunit a were able to grow on succinate as sole carbon and energy source, revealing a functional ATP synthase, in contrast to those carrying the original FLAG epitope at the same position. Binding studies with membrane vesicles of different orientation and anti-FLAG Ig demonstrated that both termini of the protein are located at the cytoplasmic side of the membrane, indicating that an even number of membrane-spanning segments is present in subunit a. In addition, insertion of two FLAG epitopes in tandem after K66, or one epitope after H95, and Q181 revealed that the polypeptide regions including these residues are accessible from the cytoplasmic surface of the membrane. These results support the view that the polypeptide chain of subunit a traverses the membrane six times.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 9492276 [PubMed - indexed for MEDLINE]

☐ **28:** Biochem J. 1997 Aug 1;325 (Pt 3):693-700. Related Articles, Links

BIOCHEMICAL JOURNAL full text article in PubMed Central

Evidence for the formation of a heterotrimeric complex of leukaemia inhibitory factor with its receptor subunits in solution.

Zhang JG, **Owczarek CM**, **Ward LD**, **Howlett GJ**, **Fabri LJ**, **Roberts BA**, **Nicola NA**.

The Walter and Eliza Hall Institute of Medical Research and The Cooperative Research Centre for Cellular Growth Factors, P.O. Royal Melbourne Hospital, Victoria 3050, Australia.

Leukaemia inhibitory factor (LIF) is a polyfunctional cytokine that is known to require at least two distinct receptor components (LIF receptor alpha-chain and gp130) in order to form a high-affinity, functional, receptor complex. Human LIF binds with unusually high affinity to a naturally occurring mouse soluble LIF receptor alpha-chain, and this property was used to purify a stable complex of human LIF and mouse LIF receptor alpha-chain from pregnant-mouse serum. Recombinant soluble human gp130 was expressed, with a FLAG(R) epitope (DYKDDDDK) at the N-terminus, in the methylotropic yeast Pichia pastoris and purified using affinity chromatography. The formation of a trimeric complex in solution was established by native gel electrophoresis, gel-filtration chromatography, sedimentation equilibrium analysis, surface plasmon resonance spectroscopy and chemical cross-linking. The stoichiometry of this solution complex was 1:1:1, in contrast with that of the complex of interleukin-6, the interleukin-6-specific low-affinity receptor subunit and gp130, which is 2:2:2.

Publication Types:

- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, P.H.S.

PMID: 9271090 [PubMed - indexed for MEDLINE]

☐ **29:** Biochem J. 1997 Jul 15;325 (Pt 2):351-7. Related Articles, Links

Localization of human heparan glucosaminyl N-deacetylase/N-sulphotransferase to the trans-Golgi network.

Humphries DE, **Sullivan BM**, **Aleixo MD**, **Stow JL**.

Department of Veterans Affairs Medical Center, Department of Medicine, Harvard Medical School, Boston, MA 02130, USA.

In order to determine the intracellular location of heparan N-deacetylase/N-sulphotransferase, cDNAs encoding human heparan glucosaminyl N-deacetylase/N-sulphotransferase were cloned from human umbilical vein endothelial cells. The deduced amino acid sequence was identical to that of the human heparan N-sulphotransferase cloned previously [Dixon, Loftus, Gladwin, Scambler, Wasmuth and Dixon (1995) Genomics 26, 239-244]. RNA blot analysis indicated that two heparan N-sulphotransferase transcripts of approx. 8.5 and 4 kb were produced in all tissues. Expression was most abundant in heart, liver and pancreas. A cDNA encoding a Flag-tagged human heparan N-sulphotransferase (where Flag is an epitope with the sequence DYKDDDDK) was transfected into mouse LTA cells. Immunofluorescence detection using anti-Flag monoclonal antibodies demonstrated that the enzyme was localized to the trans-Golgi network. A truncated Flag-tagged heparan N-sulphotransferase was also retained in the Golgi, indicating that, as for many other Golgi enzymes, the N-terminal region of heparan N-sulphotransferase is sufficient for retention in the Golgi apparatus.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, Non-P.H.S.
- Research Support, U.S. Gov't, P.H.S.

PMID: 9230113 [PubMed - indexed for MEDLINE]

☐ **30:** Can J Physiol Pharmacol. 1997 Jun;75(6):690-5. Related Articles, Links

Epitope tagging and immunoreactivity of the human angiotensin II type 1 receptor.

Roy SF, **Laporte SA**, **Escher E**, **Leduc R**, **Guillemette G**.

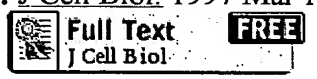
Department of Pharmacology, Faculty of Medicine, Universite de Sherbrooke, QC, Canada.

Structural analysis of G-protein-coupled receptors has largely been limited to photoaffinity labeling and site-directed mutagenesis. This is primarily due to the difficulty in the production of antibodies against this class of receptors. We were therefore interested in tagging the amino-terminal side of the human angiotensin II (AngII) type 1 receptor (AT1) with the FLAG epitope DYKDDDDK. Competitive binding experiments with [125I][Sar1,Ile8]AngII revealed that stably transfected Chinese hamster ovary (CHO) cells express 37,300 hAT1-FLAG receptors/cell with a high affinity of 0.53 nM, comparable with that of the wild-type hAT1. After photolabeling and solubilization, a significant proportion of hAT1-FLAG specifically immunoprecipitated with anti-FLAG M5 and M2 antibodies. The immunoprecipitated receptor comigrated on SDS-PAGE with photolabeled wild-type hAT1. Immunofluorescence studies by FACS scan analysis revealed that 11.9% of CHO cells expressing hAT1-FLAG receptor significantly increased their fluorescence level as a result of M5 specific reactivity. Western blot analysis failed to show any specific interaction between M5 antibody and denatured hAT1-FLAG receptor. These results demonstrate the efficiency of the epitope tagging approach for specific immunoreactivity against AT1 receptor. Appropriate refinements of this approach could improve the level of immunoreactivity.

Publication Types:
- Research Support, Non-U.S. Gov't

PMID: 9276150 [PubMed - indexed for MEDLINE]

☐ **31:** J Cell Biol. 1997 Mar 10;136(5):1037-45. Related Articles, Links



Topology of the Shaker potassium channel probed with hydrophilic epitope insertions.

Shih TM, Goldin AL.

Department of Microbiology and Molecular Genetics, University of California, Irvine 92697-4025, USA.

The structure of the Shaker potassium channel has been modeled as passing through the cellular membrane eight times with both the NH2 and COOH termini on the cytoplasmic side (Durrell, S.R., and H.R. Guy. 1992. Biophys. J. 62:238-250). To test the validity of this model, we have inserted an epitope consisting of eight hydrophilic amino acids (DYKDDDDK) in predicted extracellular and intracellular loops throughout the channel. The channels containing the synthetic epitope were expressed in Xenopus oocytes, and function was examined by two-electrode voltage clamping. All of the mutants containing insertions in putative extracellular regions and the NH2 and COOH termini expressed

functional channels, and most of their electrophysiological properties were similar to those of the wild-type channel. Immunofluorescent staining with a monoclonal antibody against the epitope was used to determine the membrane localization of the insert in the channels. The data confirm and constrain the model for the transmembrane topology of the voltage-gated potassium channel.

Publication Types:
- Research Support, Non-U.S. Gov't
- Research Support, U.S. Gov't, Non-P.H.S.
- Research Support, U.S. Gov't, P.H.S.

PMID: 9060468 [PubMed - indexed for MEDLINE]

☐ **32:** Mol Divers. 1997;2(3):156-64. Related Articles, Links

SpringerLink FULL-TEXT ARTICLE

Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5.

Slootstra JW, **Kuperus D**, **Pluckthun A**, **Meloen RH**.

Department of Molecular Recognition, Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands.

The FLAG peptides DYKDDDDK and MDYKDDDDK are widely used affinity tags. Here we describe new variants of the FLAG peptides which, in direct ELISA, showed selective and differential binding to the commercially available anti-FLAG monoclonal antibodies M1, M2 and M5. Variants of the FLAG peptides were synthesized on polymer-grafted plastic pins, and in an ELISA incubated with mAbs M1, M2 and M5. Among the newly identified tag sequences are those that bind only one of the anti-FLAG mAbs and those that bind only two or all three of the anti-FLAG mAbs. Examples of new tag sequences are MDFKDDDDK (which binds mAb M5 and does not bind mAbs M1 and M2) and MDYKAFDNL (which binds mAb M2 and does not bind mAbs M1 and M5). The sensitivity in direct ELISA of some variants was increased, e.g. using mAb M2 it was found that replacing DDDDK in MDYKDDDDK by AFDNL increased the sensitivity in ELISA at least 10-fold. The activity of this peptide was studied in more detail. In different direct ELISAs, in which MDYKAFDNL was synthesized on polyethylene pins, coated onto polystyrene microtiter plates or onto nitrocellulose paper, the activity of this peptide was similar, i.e. increased at least 10-fold over that of MDYKDDDDK. Remarkably, in competitive ELISA the binding activity of soluble MDYKAFDNL was decreased 10-fold over those of soluble MDYKDDDDK or DYKDDDDK. The results seem to suggest that, in solution, the conformation of MDYKAFDNL is more 'unstructured' compared to its conformation when coated or linked to a carrier. We postulate that the newly described tag sequences may be used as affinity tags to separately detect, quantify and purify multiple co-expressed proteins and/or

subunits.

Publication Types:
- In Vitro
- Research Support, Non-U.S. Gov't

PMID: 9238646 [PubMed - indexed for MEDLINE]

☐ **33:** J Virol. 1996 Oct;70(10):6831-8. Related Articles, Links

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Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies.

Wickham TJ, Segal DM, Roelvink PW, Carrion ME, Lizonova A, Lee GM, Kovesdi I.

GenVec, Inc., Rockville, Maryland 20852, USA.

A major hurdle to adenovirus (Ad)-mediated gene transfer is that the target issue lacks sufficient levels of receptors to mediate vector attachment via its fiber coat protein. Endothelial and smooth muscle cells are primary targets in gene therapy approaches to prevent restenosis following angioplasty or to promote or inhibit angiogenesis. However, Ad poorly binds and transduces these cells because of their low or undetectable levels of functional Ad fiber receptor. The Ad-binding deficiency of these cells was overcome by targeting Ad binding to alpha v integrin receptors that are sufficiently expressed by these cells. In order to target alpha v integrins, a bispecific antibody (bsAb) that comprised a monoclonal Ab to the FLAG peptide epitope, DYKDDDDK, and a monoclonal Ab to alpha v integrins was constructed. In conjunction with the bsAb, a new vector, AdFLAG, which incorporated the FLAG peptide epitope into its penton base protein was constructed. Complexing AdFLAG with the bsAb increased the beta-glucuronidase transduction of human venule endothelial cells and human intestinal smooth muscle cells by seven- to ninefold compared with transduction by AdFLAG alone. The increased transduction efficiency was shown to occur through the specific interaction of the complex with alpha v integrins. These results demonstrate that bsAbs can be successfully used to target Ad to a specific cellular receptor and thereby increase the efficiency of gene transfer.

PMID: 8794324 [PubMed - indexed for MEDLINE]

☐ **34:** J Biol Chem. 1995 Dec 22;270(51):30818-22. Related Articles, Links

Full Text FREE J Biol Chem

The guanylyl cyclase-A receptor transduces an atrial

natriuretic peptide/ATP activation signal in the absence of other proteins.

Wong SK, Ma CP, Foster DC, Chen AY, Garbers DL.

Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas 75235, USA.

Attempts to activate partially purified preparations of the guanylyl cyclase-A (GC-A) receptor with atrial natriuretic peptide (ANP) have previously failed, leading to speculation that essential cofactors are lost during purification procedures. The receptor was modified to contain the FLAG epitope (DYKDDDDK), expressed in Sf9 cells, and purified to apparent homogeneity (4.3 mumol cyclic GMP formed/min/mg protein; 5.8 mmol 125I-ANP binding site/mg protein) by a combination of immunoaffinity, Q-Sepharose FF, and wheat germ agglutinin batch chromatography. High initial protein/detergent ratios, the presence of glycerol (40%), and the inclusion of protein phosphatase inhibitors in all buffers resulted in the purification of a receptor that continued to transduce the ANP/ATP activation signal. Both native and purified GC-A contained a single class of high affinity ANP binding sites (Kd = 60 pM) and an equivalent EC50 for ATP (0.3 mM). Positive cooperativity as a function of MnGTP was retained during purification. Thus, GC-A is capable of transducing a ligand binding signal in the absence of other proteins.

Publication Types:
- Research Support, U.S. Gov't, P.H.S.

PMID: 8530525 [PubMed - indexed for MEDLINE]

☐ **35:** Mol Divers. 1995 Sep;1(1):21-8. Related Articles, Links

Mapping the detailed specificity of a calcium-dependent monoclonal antibody through the use of soluble positional scanning combinatorial libraries: identification of potent calcium-independent antigens.

Pinilla C, Buencamino J, Appel JR, Hopp TP, Houghten RA.

Torrey Pines Institute for Molecular Studies, San Diego, CA 92121, USA.

The detailed specificity of monoclonal antibody M1, which has been reported to bind in a calcium-dependent manner to the 'FLAG' sequence DYKDDDDK-NH2, was examined using soluble hexa- and decapeptide positional scanning synthetic combinatorial libraries (PS-SCLs) made up of 52 x 10(6) and 4 x 10(12) different sequences, respectively. To study the influence of calcium on the specificity of this antigen-antibody interaction, each PS-SCL was screened in the presence and absence of calcium using a competitive ELISA. Overall, peptide mixtures had greater inhibitory activity against mAb M1 binding to FLAG in the

absence of calcium. A total of 16 individual hexapeptides were identified, all of which contained the motif-DYK_K_(-), and were recognized by mAb M1 in the absence of calcium with 50- to 100-fold higher affinity than the FLAG octapeptide (IC50 = 273 nM). On average, the same set of peptides bound 10-fold less effectively in the presence of calcium. Upon screening the decapeptide PS-SCL in the absence of calcium, lysine was also more active in the fifth position than the original aspartic acid. Based on the screening results, 24 individual decapeptides were prepared and were found to have activities 10- to 100-fold higher than the FLAG octapeptide in the absence of calcium. The specificity of lysine at the fifth position in the antigen-antibody interaction was further examined by synthesizing and assaying substitution analogs at this position for the octapeptide and hexapeptide forms of the FLAG sequence, as well as for two hexapeptides identified from the PS-SCL. Truncation analog analysis was also carried out on the FLAG octapeptide to determine optimal antigen length for antibody binding. Overall, lysine at the fifth position could be substituted with ornithine with no significant loss in activity, and peptide length was not a critical factor for antibody binding in the absence of calcium. Also, the octapeptide having lysine at the fifth position in place of the aspartic acid had the same activity in the presence or absence of calcium. This study demonstrates the ease and effectiveness of PS-SCLs over individual peptide analogs for the examination of the degree of cross-reactivity for a given monoclonal antibody as well as for the identification of novel, high-affinity peptides.

Publication Types:

- In Vitro
- Research Support, Non-U.S. Gov't

PMID: 9237191 [PubMed - indexed for MEDLINE]

☐ **36:** Biotechniques. 1994 Oct;17(4):754-61. Related Articles, Links

An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments.

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The commercially available monoclonal antibodies M1 and M2 were raised against and bind the FLAG sequence DYKDDDDK with high specificity. Using the calcium-dependent M1 antibody and the FLAG tag attached to the N terminus of various fragments of the antibody McPC603 expressed in Escherichia coli, we found that the M1 antibody binds with almost the same affinity to a much shorter version of this sequence (DYKD). Since most antibody light chains start with an aspartate, the addition of only three additional amino acids to the N terminus is sufficient to detect and quantify the expressed antibody fragments using standard immunological methods. Similarly, the heavy

chain can be detected specifically with the sequence DYKD, which requires four additional amino acids since most heavy chains do not start with Asp. The signal sequence of both chains that is necessary for the transport of the chains to the periplasm of E. coli is processed correctly. Furthermore, we investigated the influence of the amino acid at the fifth position of the FLAG sequence on the binding affinity of the M1 antibody and found that a glutamate at this position increased the sensitivity in Western blots sixfold over the original long FLAG sequence containing an aspartate residue at this position. Together, the improved FLAG is a versatile tool for both sensitive detection and one-step purification of recombinant proteins.

Publication Types:
- Research Support, Non-U.S. Gov't

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☐ **37:** J Neurosci. 1994 Feb;14(2):834-45. Related Articles, Links

Full Text J Neurosci

Characterization of antibodies to the rat substance P (NK-1) receptor and to a chimeric substance P receptor expressed in mammalian cells.

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Antibodies to neuropeptide receptors can be used to localize and characterize the receptors in tissues and cell lines. Two strategies were used to study the rat substance P receptor (SPR, NK-1) by immunological methods. First, a polyclonal antiserum was raised by immunizing rabbits with a peptide corresponding to the 15 amino acid residues (KTMTESSSFYSNMLA, SPR393-407) at the intracellular C-terminus of the rat SPR coupled to bovine thyroglobulin. An antiserum was obtained with a titer for half-maximal binding of 125I-SPR393-407 of 1:70,000. Nonradioactive SPR393-407 inhibited 50% of binding at a concentration of 10 pM. Binding of 125I-SPR393-407 to the antiserum was also displaced in a parallel manner by membrane proteins from tissues expressing high levels of the SPR (brain and submaxillary gland). Second, a chimeric SPR construct of a hydrophilic Flag peptide (DYKDDDDK) genetically engineered in sequence with the extracellular N-terminus of rat SPR was generated by polymerase chain reaction. The Flag-SPR chimera was expressed in rat kidney epithelial cells (KNRK) and judged to be fully functional, assessed by binding of 125I-substance P (apparent Kd of 5.63 nM) and calcium mobilization in response to substance P (EC50 of 0.66 nM). Antibodies to SPR393-407 and the Flag peptide stained the plasma membrane of KNRK cells expressing the native SPR or the Flag-SPR chimera. Staining was abolished by preincubation with SPR393-407 or the Flag peptide. Cells

transfected with vector alone were unstained. The SPR antiserum recognized a broad protein band on Western blots of membranes prepared from cells expressing SPR but not from cells transfected with vector alone. The signal was quenched by preincubation of the antiserum with SPR393-407. By immunohistochemistry, the SPR antiserum was found to bind to neurons in the dorsal horn of the rat spinal cord and to ganglion cells in the myenteric plexus of the rat ileum near substance P-immunoreactive nerve fibers. Staining was abolished by preabsorption of the antiserum with SPR393-407. These antibodies can be used to localize the SPR in tissues and cells and to examine the function of the receptor in cell lines.

Publication Types:

- Research Support, U.S. Gov't, P.H.S.

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